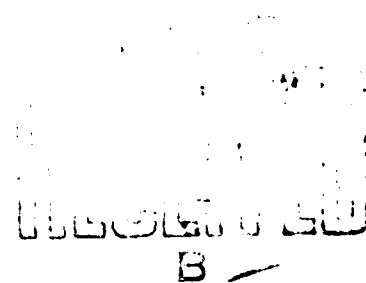


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The Original Haemorrhagic Fever: Yellow Fever

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SUMMARY. Monkeys infected with the Asibi strain of yellow fever virus developed multiple coagulation abnormalities 3–4 days after viral inoculation. These coagulation abnormalities occurred almost simultaneously with the development of fever, clinical illness and pathologic changes in the liver. The haemorrhagic manifestation of yellow fever and the rapid development of multiple coagulation abnormalities cannot be explained solely by impaired production of coagulation proteins and suggested that there was accelerated intravascular coagulation. Heparin therapy in one group of infected animals decreased the severity of coagulation abnormalities but did not prolong the life of these animals.

Yellow fever, a group B arbo virus infection, varies in severity from a mild febrile illness to a fulminant infection characterized by hepatic failure, albuminuria, haemorrhagic manifestations, coma and death. Often the terminal event in fatal cases is an upper gastro-intestinal haemorrhage, classically described as 'black vomitus'. The generalized bleeding tendency may also be manifested by epistaxis, bleeding into the mucous membranes and uterine haemorrhage (Strode, 1951; Horsfall and Tamm, 1965).

The prominence of haemorrhagic manifestations in severe cases of yellow fever prompted this investigation of coagulation abnormalities in monkeys infected with a virulent strain of yellow fever virus.

MATERIALS AND METHODS

Thirty-two rhesus (*Macaca mulatta*) monkeys weighing 2.5–3.5 kg. were used for this study. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed. All monkeys were bled 1 week prior to infection for baseline coagulation studies and measurement of yellow fever virus neutralizing antibody titres. Twenty-four of the monkeys were inoculated intraperitoneally with 1 ml. of rhesus monkey serum containing 10^3 mouse intracerebral (i.c.) median lethal doses of Asibi strain of yellow fever virus.

Virus assays were performed by intracerebral inoculation in weanling mice (Collier, De Roever-Bonnet and Hoekstra, 1959).

Coagulation studies were carried out on each infected monkey prior to infection and on one of the four days after inoculation and again when they were gravely ill. Control studies were performed on unaffected control animals and on 24 normal rhesus monkeys. Four monkeys

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were injected with 250 units of heparin sodium (1000 NIH units/ml.) intraperitoneally every 6 hours from 24 hours after virus inoculation until death. Further tests were performed on blood obtained from these latter animals 4 days after infection and compared with preinfection specimens and with blood obtained from unheparinized infected monkeys.

The blood for these studies was collected in disposable polypropylene syringes with 20 gauge needles by femoral venipuncture. Citrated (3.8 per cent sodium citrate) and oxalated (0.1 M sodium oxalate) specimens were prepared by mixing 4.5 ml. of whole blood with a 0.5 ml. of anticoagulant. Platelet-poor plasma was prepared by centrifuging the oxalated and citrated specimens at 3500 rev./min. at 4° C. for 5 minutes. The oxalated and citrated platelet-poor plasmas were pipetted into separate polypropylene tubes with disposable polypropylene pipettes and rapidly frozen in alcohol and dry ice. These specimens were stored at -70° C. until clotting studies were performed. Whole blood anti-coagulated with dry ethylene diaminetetracetate (EDTA) was used for the determination of routine haematologic studies including platelet counts. An additional specimen of whole blood was collected in a clean, dry test tube, allowed to clot and incubated at 37° C. for 4 hours. These specimens were centrifuged at 5000 rev./min. for 20 minutes, the serum decanted into a clean polypropylene test tube and frozen at -70° C. until used for the determination of fibrinogen degradation products. Normal values for coagulation data were obtained by studies of the blood from 24 normal rhesus monkeys. The plasma from these animals was prepared and frozen similarly to specimens from the test monkeys.

The one-stage prothrombin time was measured by the method of Quick (1935) using rabbit brain thromboplastin (Ortho Diagnostic Division, Ortho Pharmaceutical Co.).

The partial thromboplastin time was performed by the method of Langdell, Wagner and Brinkhous (1953), using rabbit brain cephalin with celite as the plasma activator (Bioquest, Division of Baltimore Biological Lab.).

Prothrombin (Factor II) activity was determined by the method of Owren (1949).

Labile factor (Factor I') activity was determined by a modification of the method of Stefanini (1950).

Stable factor (Factor VII) and Stuart Prower factor (Factor X) activities were assayed by the method of Owren and Aas (1951).

Antihæmophilic globulin (Factor VIII) activity was determined by the method of Bergna (1960).

Plasma thromboplastin component (Factor IX) was measured by the method of Kruptakin, Hoag and Aggeler (1964).

Stuart-Prower factor (Factor X) activity was determined by the method of Bachman, Duckert and Koller (1958).

Plasma thromboplastin antecedent (Factor XI) activity was assayed by the method of Horowitz, Wilcox and Fujimoto (1963).

Platelets were counted by the method of Rees and Ecker (Wintrobe, 1967).

Fibrinogen was measured by the method of Ratnoff and Menzie (1951).

Euglobulin lysis time (ELT) was performed by the method of Dennis and Conrad (1968).

All assays and studies requiring fibrin formation as an end point were performed with a fibrometer clot timer.

Fibrinogen degradation products (FDP). Immunodiffusion studies employing specific

rabbit antiserum against human fibrinogen prepared by a modification of the method of Hemker, Fekkes, Hensen, Schrijver and Loeliger (1966) were performed by the double diffusion method of Ouchterlony (1962) on agar plates. Test sera and normal monkey sera were treated with 100 units of bovine thrombin (1000 NIH units per ml.) and incubated for

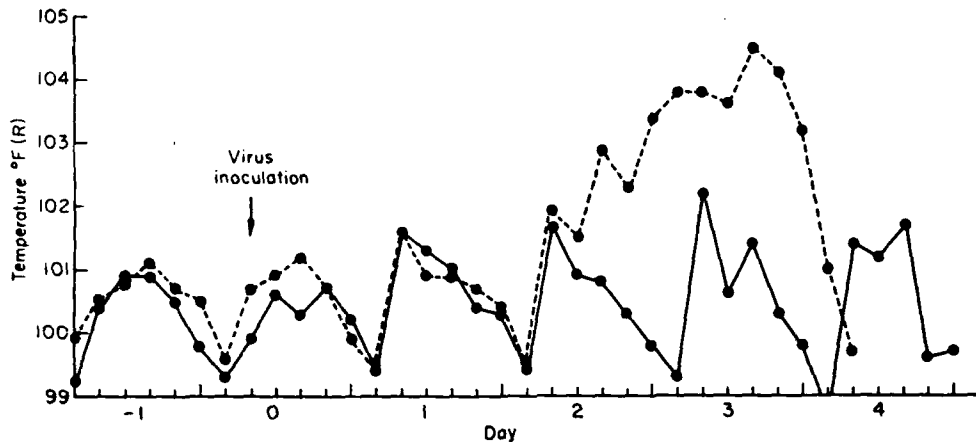


FIG. 1. Rectal temperatures of infected (---) and uninfected (—) monkeys were obtained with indwelling thermocouples. Significant variation from normal daily temperature response was observed 56 hours after inoculation with yellow fever virus.

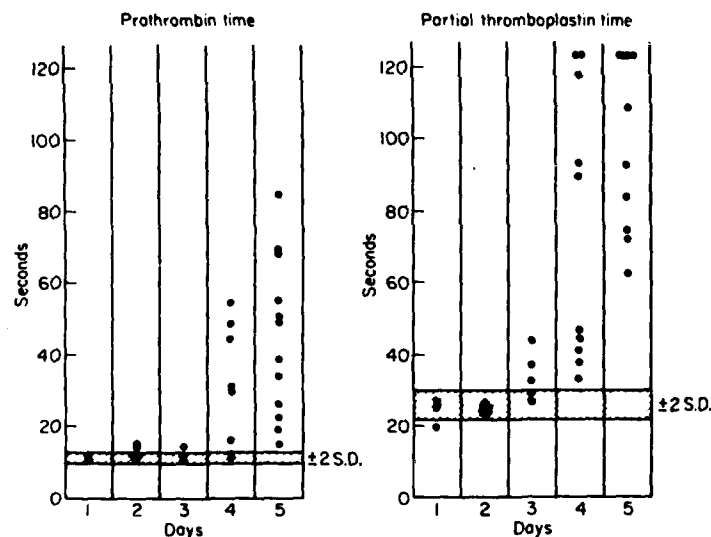


FIG. 2. The one-stage prothrombin time and partial thromboplastin time at intervals after infection of rhesus monkeys with yellow fever virus. Normal mean values ± 2 S.D. are crosshatched.

1 hour at 37°C. before utilization in the immunodiffusion studies (Merskey, Johnson and Kleiner, 1965). Similarly-treated serum from 24 normal monkeys showed no reaction of identity for fibrinogen.

Rectal temperature was measured using an indwelling thermocouple connected to a Honeywell Temperature Recorder.

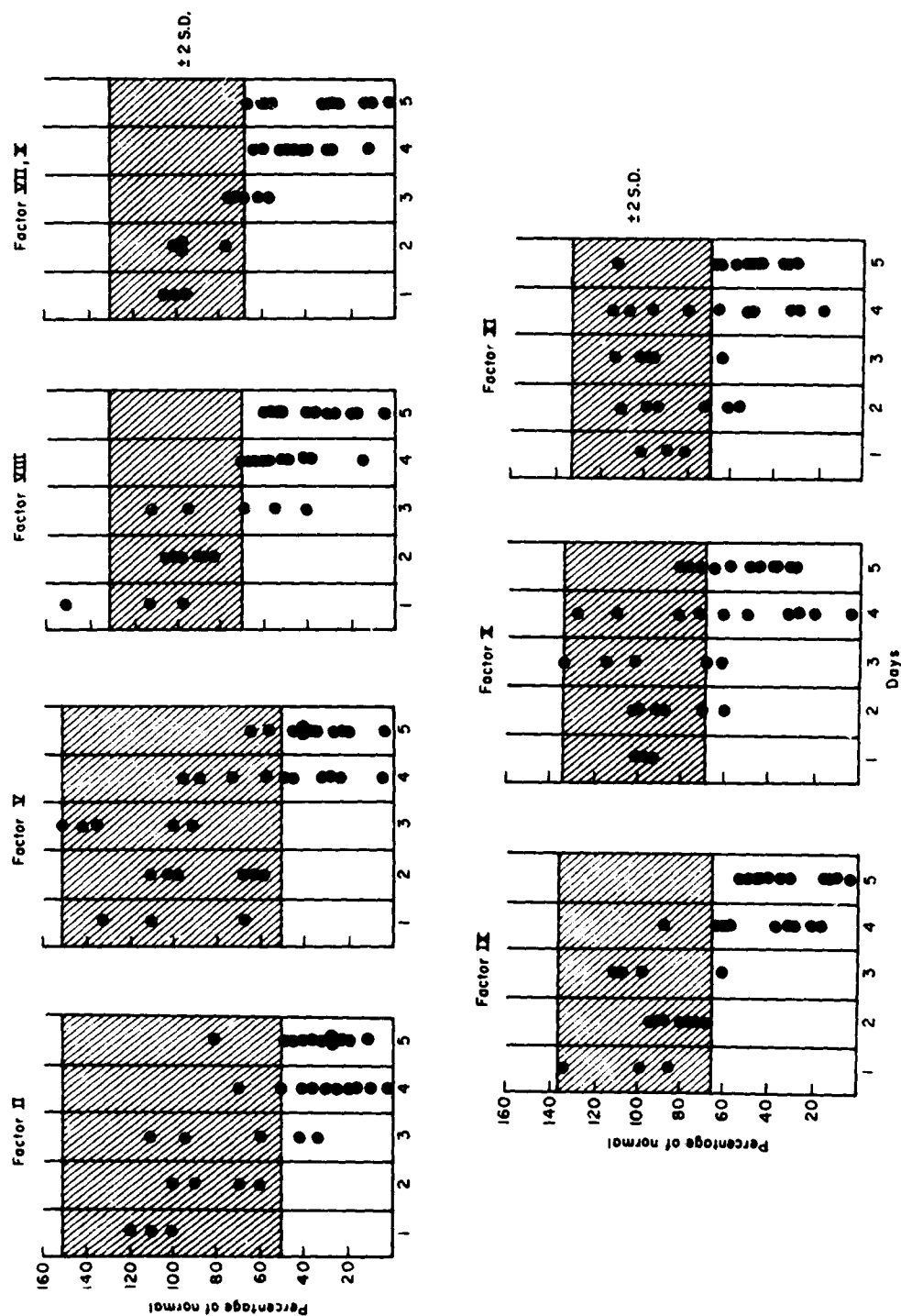


FIG. 3. Coagulation factor assays in rhesus monkeys at intervals after infection with yellow fever virus are plotted as a percentage of mean value of determinations made on blood specimens from 24 normal rhesus monkeys. Normal values ± 2 S.D. are crosshatched.

RESULTS

Rhesus monkeys, without serum-neutralizing antibodies for yellow fever virus, were injected with a lethal dose of Asibi strain of yellow fever virus. Fever became apparent during the third day of observation (Fig. 1). All infected monkeys died within 5–6 days after virus inoculation. At autopsy, the liver showed severe hepatic necrosis similar to that previously reported in rhesus monkeys with yellow fever (Tigertt, Berge, Gochenour, Gleiser, Eveland, Vorder-Bruegge and Smetana, 1960; Bearcroft, 1957). There were petechial haemorrhages in many organs and partially digested blood within the intestinal lumen of most animals. Organized thrombi were not seen by light microscopic examination of visceral organs.

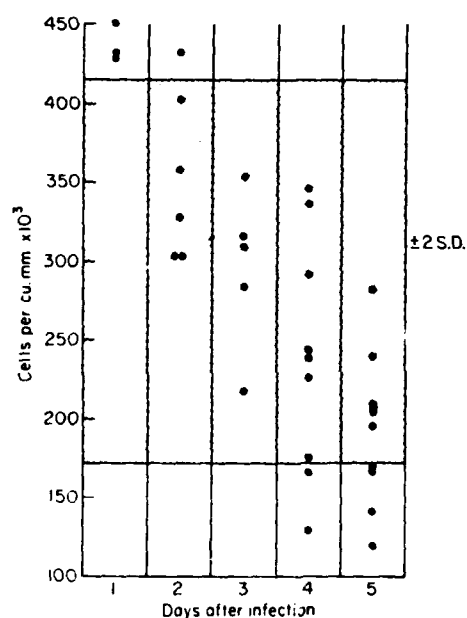


FIG. 4. Platelet counts in rhesus monkeys at intervals after infection with yellow fever virus. Normal values ± 2 S.D. are cross-hatched.

Multiple coagulation abnormalities were not observed until 72 hours after virus inoculation. As the disease progressed, infected monkeys developed a prolonged one-stage prothrombin time and partial thromboplastin time (Fig. 2). These abnormal screening studies reflected significant depression of Factors II, V, VII plus X, VIII, X, and XI (Fig. 3). The platelet counts of most monkeys remained within the normal range. However, the animals had a gradual decrease in the platelet count throughout the period of infection with the development of a mild thrombocytopenia on the fourth and fifth day (Fig. 4). The euglobulin lysis time was prolonged in half of the observed animals on the second day after infection and was abnormal in all but one specimen at later intervals (Table I). The fibrinogen concentration remained normal in blood from most monkeys, except terminally when several animals had hypofibrinogenaemia (Fig. 5). This, together with the relatively prolonged thrombin times (Fig. 5)

may be explained by the accumulation of fibrinogen degradation products (FDP) (Table II) in the circulating blood of infected monkeys.

Heparinization seemed to decrease the severity of coagulation abnormalities caused by yellow fever but did not prolong the lifespan of infected monkeys (Table III).

TABLE I
EUGLOBULIN LYSIS TIME

Lysis times	Days after infection				
	1	2	3	4	5
No. increased > 180 min.	0	3 (1, > 360)	5 (4, > 360)	9 (8, > 360)	11 (10, > 360)
No. normal 45-80 min.	3	3	0	1	0
No. decreased < 45 min.	0	0	0	0	0
Total tested	3	6	5	10	11

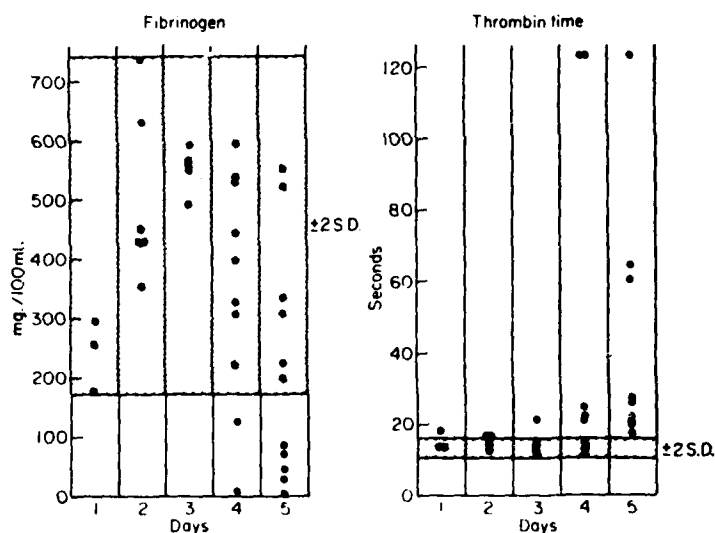


FIG. 5. Plasma fibrinogen concentration and thrombin time in rhesus monkeys at intervals after infection with yellow fever virus. Normal values ± 2 S.D. are crosshatched.

DISCUSSION

For many years, haemorrhage has been recognized as an ominous prognostic sign in patients with yellow fever. More recently, coagulation studies in humans with yellow fever showed a prolonged one-stage prothrombin time in most patients, an abnormal bleeding and clotting time in some patients and normal blood fibrinogen levels with a low normal platelet count in a few selected cases. These investigators related the abnormal prothrombin time to

liver damage and the degree of abnormality to the severity of the disease (Trejos and Romero, 1954; Elton, Romero and Trejos, 1955).

Monkeys infected with a virulent strain of yellow fever developed multiple first and second stage coagulation abnormalities and increased amounts of fibrinogen degradation products appeared in the circulating blood. This multiplicity of coagulation defects and the rapidity of their occurrence cannot be attributed solely to diminished production of clotting proteins and may be caused by accelerated utilization (McKay, 1964). The presence of fibrinogen degradation products in the circulating blood suggested that there was continuing

TABLE II
FIBRINOGEN DEGRADATION PRODUCTS IN MONKEYS INFECTED WITH YELLOW FEVER*

	Days after infection				
	1	2	3	4	5
Negative results	3	6	5	3	3
Positive results	0	0	0	7	8
Total tested	3	6	5	10	11

* Fibrinogen degradation products were sought in immunodiffusion studies utilizing thrombin-treated serum and specific rabbit antisera against human fibrinogen. No positive reactions were observed in sera from 24 normal rhesus monkeys.

TABLE III
HEPARIN THERAPY IN EXPERIMENTAL YELLOW FEVER LABORATORY TESTS IN SIX ANIMALS
BEFORE AND 4 DAYS AFTER INFECTION

	Unheparinized				Heparin treated							
	A		B		1		2		3		4	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Fibrinogen (mg./100 ml.)	749	117	463	34	388	309	599	1170	328	487	243	454
Platelets ($\times 10^{-3}$ /cu.mm.)	502	196	446	214	248	122	244	206	286	152	366	260
Fibrinogen degradation products	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg

active fibrinolysis. This finding and the prolonged euglobulin lysis time in animals with evidence of intravascular coagulation suggested that this plasmin was most likely due to plasminogen depletion as the result of continuing activator activity, plasmin production and persisting fibrinolysis in the blood or tissues.

Similar to the occurrence of a consumptive coagulopathy in other infectious diseases, laboratory abnormalities were observed almost simultaneously with the onset of fever, clinical symptoms and pathological changes in various body organs (McKay, 1964). The pathogenesis of the coagulation abnormalities is not known, but it is possible that the virus.

viral antibodies or cellular products of tissue damage could promote active intravascular coagulation.

Evidence of accelerated intravascular coagulation in monkeys with yellow fever suggested that anticoagulation therapy might reduce the severity and mortality of the disease. Heparin therapy seemed to diminish the severity of coagulation abnormalities but did not delay or prevent death. This suggested that liver necrosis and not disseminated intravascular coagulation was the cause of death in our animals. However, anticoagulants might play a more important therapeutic role in less virulent infections with yellow fever.

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